#5 8/2/02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Konowalchuk, et al.	)	
Serial No.:	10/016,282	)	Art Unit: 1617
Filed:	December 6, 2001	)	
Title:	METHODS FOR PREVENTING LESIONS CAUSED BY VIRUSES OF THE HERPESVIRIDAE OR POXVIRIDAE FAMILY	)	Examiner: Hui, S.

To: Commissioner for Patents
Washington, D.C. 20231

RULE 132 DECLARATION OF DR. JACK KONOWALCHUK

STATE OF OREGON)
) ss.
COUNTY OF LINCOLN)

I, Jack Konowalchuk, declare:

1. My name is Jack Konowalchuk, and I reside at 1098 N.E. 7th Drive, Newport, Oregon.

2. All of my statements in this Declaration are accurate and true to the best of my knowledge and belief.

3. I am currently a research scientist, a position I have held since 1999, where I am responsible for research activities on the virucidal composition.

4. As background information and as foundation for my statements in this Affidavit, I received a Bachelor of Science Degree from University of Manitoba in 1946, and I received the degree of Doctorate in Microbiology from the Queen's University in 1952. I have over 50 years of experience researching in the virology field.

5. From 1947-1963 at the Defense Research Kingston Laboratories, I was the Scientific Officer respondent for general microbial research.

6. In 1963, I accepted a position with Defense Research Board, Shirley's Bay

Ottowa as Head of Virus group as Scientific Officer responsible for the rapid identification of human viruses.

7. From 1969-1985, I worked at Health and Welfare Canada, Health Protection Branch, Bureau of Microbial Hazards as Head of Food Virology, Research Scientist 3. I planned an developed methods for virus recovery from foods.

8. I have authored or co-authored more than 35 scientific publications in my career and I have presented many papers at numerous proceedings.

9. I am a co-inventor of U.S. Patent Application No. 09/795,279, filed February 28, 2001.

10. I have reviewed the Examiner's final Office Action, Paper No. 10, dated February 12, 2002. It is my understanding the Examiner has determined that the data presented in U.S. Patent Application No. 09/795,279 do not show unexpected results, and that it would have been obvious to combine the agents of the claimed compositions based on the prior art cited in the final Office Action. I do not agree.

11. I have reviewed the references cited in Exhibit B to be submitted with the response to the final Office action.

12. It is my belief that the references cited in Exhibit B demonstrate that lower chain alcohols at low concentrations alone do not have virucidal activity.

13. I conducted the assays summarized in Table 2 of U.S. Patent Application No. 09/795,279.

14. The data presented in Table 2 of the present invention demonstrate that glycolic acid alone is virucidally effective when at a pH at or below 4.0. The data also demonstrate that glycolic acid is not virucidally effective when at a pH above 4.0.

15. I conducted the assays summarized in Exhibit C to be submitted with the response to the final Office action, Paper No. 10.

16. The data provided in Exhibit C summarizes the results of an assay in which various amounts of ethanol (1%, 5% or 10%) were combined with a 0.6% glycolic acid solution and the pH of the mixtures were adjusted to a pH of 2.5, 3.5, 4.0, 4.5 or 5.0. The final solutions were then assayed for virucidal activity against the Herpes Simplex 1 virus in a manner similar to that described in Example 1 of U.S. Patent Application No. 09/795,279.

17. Exhibit C provides supporting data showing the synergistic effect of the acid, alcohol, and pH agents of the claimed invention. The data provided in Exhibit C show that when low concentrations of ethanol (e.g., 1-10%) are combined with glycolic acid at a pH between 2.5 and 4.5, the resulting compositions have virucidal activity. However, when the pH is at 4.5 or above, the compositions have little or no virucidal activity.

18. I believe that the data provided in U.S. Patent Application No. 09/795,279 and in Exhibits B and C demonstrate that lower chain alcohols at low concentrations are not virucidal and also that acid solutions above pH 4.5 are not virucidal, but the combination of lower chain alcohols at low concentrations and an acid at a pH between 2.45 and 4.6 provides an effective virucidal composition. Therefore, this combination of non-virucidal agents to produce a virucidal composition demonstrates a synergistic effect.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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0.6 % glycolic acid mixed with various amounts of ethanol (10 min exposure)

	pH				
% Ethanol	2.5	3.5	4.0	4.5	5.0
1%	-	-	-	+	+
5%	-	-	-	-	+
10%	-	-	-	-	-*

- : No virus growth
+ : Virus growth

* One of two plates had no virus, the other had only one plaque.
Typical plates for compositions that are not active have at least 10 plaques.

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The action of alcohols on rotavirus, astrovirus and enterovirus

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Summary: The virucidal effects of a series of five alcohols on rotavirus, astrovirus and echovirus 11 were studied. The reaction time between the alcohol and virus was one minute, a time for which a hand disinfectant might be applied. The efficacy of the alcohols rose with the concentration used. Forty per cent concentrations of the higher alcohols (propan-1-ol, propan-2-ol and butan-2-ol) caused at least a 10^4-fold drop in rotavirus titre. Methanol and ethanol were not quite as effective against rotavirus, but were the only alcohols of those tested that reduced the titres of the more resistant astrovirus and echovirus 11, and then only when used at high concentrations. Preparations incorporating 90 per cent ethanol are recommended as hand disinfectants with a broad virucidal activity.

Introduction

Much has been written on the action of disinfectants against pathogenic bacteria and standard procedures are now used to evaluate them. For disinfecting skin Lowbury, Lilly & Ayliffe (1974) have clearly shown the effectiveness of an alcoholic solution of chlorhexidine in reducing the viable bacterial counts of surgeons' hands. The virucidal activity of hand disinfectants has, however, seldom been studied in spite of reports that enteroviruses (Melnick, 1976) and rhinoviruses (Hendley, Wenzel & Gwattney, 1973) may be spread on hands.

In hospitals, babies in neonatal units are especially subject to cross-infection which may be endemic, as was the the case for rotaviruses reported by Chrystie, Totterdall & Banatvala (1978) or take the form of epidemics, an example of which was the Cambridge echovirus 11 outbreak reported by Nagington, Wreghitt, Gandy, Robertson & Berry (1978). The relative effects of various disinfectants on echovirus 11 have been studied by Drulak, Wallbank & Lebtag (1978), and they found that 76 per cent (v/v) ethyl alcohol caused a 10^6 reduction in virus titre following 20 s exposure. Our studies (Kurtz, 1979), supported this finding, but also showed that isopropyl alcohol at a similar dilution failed to lower the virus count following 1 min contact, a time for which a hand disinfectant might be applied.

We report now the reduction in infectivity of rotavirus and astrovirus, both of which are common causes of infantile gastroenteritis, as well as echovirus 11, following 1 min contact with various alcohols.

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Materials and methods

The alcohols tested were methanol, ethanol, propan-1-ol, propan-2-ol and butan-2-ol (B.D.H. Chemicals Ltd).

Tests with rotavirus

An adapted strain of bovine rotavirus (kindly provided by Dr Bridger, ARC, Compton, Berkshire), grown in $LLCMK_2$ cells was ampouled and stored at −70°C. The activity of the alcohols was tested as follows: to 0·3 ml volumes of the virus containing fluid were added 0·7 ml volumes of different dilutions of the alcohols, to give final concentrations between 20 and 70 per cent. After 1 min, 2 ml of serum-free 199 medium containing 0·2 μg/ml trypsin (199T), which was incorporated into the medium to enhance the infectivity of rotaviruses in tissue culture (Almeida, Hall, Banatvala, Totterdell & Chrystie, 1978), was added to the mixture. Immediately a further 1:10 dilution of this was made in 199T. For a control, 0·3 ml of the virus containing fluid was held with 0·7 ml phosphate buffered saline (PBS) for 1 min and then after adding 2 ml of 199T the 10-fold dilution was made in 199T containing 2·5 per cent of the alcohol under test. The alcohol was added to the control at this stage to match the effect of the residual alcohol in the test which might continue to affect the virus or the cells. Virus infectivity was assayed in $LLCMK_2$ cells. 2 ml aliquots of the above dilutions were put onto coverslips in flat-bottomed tubes. The tubes were centrifuged at 3000 r/min for 1 h at 35°C. The medium was replaced with 199T and the tubes incubated at 37°C for 18–24 h. After fixing in acetone, the coverslips were treated with a bovine anti-rotavirus serum and then fluorescein labelled rabbit anti-bovine globulin (Wellcome Research Laboratories Ltd.). The coverslips were examined with a Vickers incident light fluorescent microscope and the fluorescing cells counted. From these the number of infective doses of virus in the reaction mixtures were calculated. Further tests to determine the effect of organic material on the activity of the alcohols were done using equal volumes of virus and sterilized faeces.

Tests with astrovirus

A human faecal extract (10 per cent) containing large numbers of astroviruses was used as the virus source. Virucidal activity was measured by adding to 0·1 ml volumes of the faecal extract 0·9 ml of dilutions of the alcohols under test. After one minute reaction time, 19 ml of 199 medium containing 20 per cent foetal calf serum (199S) was added, and further 10-fold dilutions made in the same medium. For a control, similar volumes of faecal extract and PBS were mixed and held for one minute after which the initial dilution was made in 199S containing 4·5 per cent of the alcohol under test. Aliquots of 1 ml of dilutions were then put onto monolayers of human embryo kidney cells grown on coverslips. After one hour the inoculum was removed and replaced by 199S. The coverslips were then incubated 18–24 h at 37°C. After fixing in acetone the coverslips were treated with a human anti-astrovirus serum, stained with a fluorescein labelled rabbit anti-human globulin (Wellcome Research Laboratories Ltd), and the fluorescing cells counted as above.

Tests with echovirus 11

A recently isolated strain of echovirus 11 grown in human embryo lung fibroblasts (HEL) and stored at −70°C was used. The method originally described by Drulak *et al.* (1978), who found that 17·5 per cent skimmed milk effectively neutralized the effect of a diverse group of disinfectants, was adapted (Kurtz, 1979), and was briefly as follows: to 0·05 ml of the virus and 0·05 ml of calf serum were added 0·4 ml volumes of dilutions of the alcohol to be tested. The concentration of alcohol in the reaction mixture was 4/5th of its initial concentration. After 1 min reaction time, 4·5 ml of skimmed milk (17·5 g per 100 ml) was added to neutralize the effect of the alcohol. A two-fold dilution and then serial 10-fold dilutions were made in Eagles MEM, supplemented with 2 per cent calf serum. Volumes of 1 ml of these dilutions were inoculated onto HEL monolayers and observed for up to 5 days for cytopathic effect (CPE). Virus titres were recorded as the greatest dilution (log_{10}) showing any CPE and the number of infective units/ml of reaction mixture calculated. For a control, to 0·1 ml of the virus–serum mixture was added 0·4 ml PBS and the skimmed milk added one minute later contained 9·5 per cent of the alcohol being tested.

Results

Table I shows that rotavirus titres were not affected by 20 per cent methanol or ethanol, although there was a 1–3 log_{10} drop when the higher alcohols were used at this concentration. With 30 per cent concentrations, all the alcohols tested, except

Table I. *Bovine rotavirus titres after alcohol treatment (1-min holding time)*

	Alcohol in reaction mixture (per cent)				
	0 (control)	20	30	40	50
Methanol	5·9*	5·5	5·7	3·6	2·2
+ faeces	5·3			3·3	2·3
Ethanol	5·9	5·9	2·7	<1·9	
+ faeces	5·7		1·9	<1·9	
Propan-1-ol	5·9	2·9	2·2	<1·9	
+ faeces	5·2		1·9		<1·9
Propan-2-ol	5·8	4·3	2·3	<1·9	
+ faeces	5·5		2·9		
Butan-2-ol	5·9	2·9	<1·9		
+ faeces	5·3		1·9	<1·9	

*Log_{10} infective units/ml reaction mixture.
Each entry is the mean of four experiments.

methanol, gave a 3–4 log_{10} reduction in titre. Rotavirus was still present after treatment with 50 per cent methanol, but the other alcohols all reduced the virus count below the minimum detectable in this test system (75 infective units/1 ml reaction mixture) when used at a 40 per cent concentration. Higher concentrations of all the alcohols (70 per cent and 90 per cent) again lowered the virus titre below detectable numbers. It can also be seen that the presence of faecal material caused only a slight reduction in the effectiveness of the alcohols.

Table II. *Astrovirus titre after alcohol treatment (1 min holding time)*

	Alcohol in reaction mixture (per cent) 0 (control)	70	90
Methanol	4·0*	1·0	<1·0
+ faeces	4·0	1·0	<1·0
Ethanol	5·7	4·3	1·7
+ faeces	4·5		3·8
Propan-1-ol	5·3		5·3
Propan-2-ol	4·8		4·7
Butan-2-ol	4·8		4·8

*Log_{10} infective units/ml of reaction mixture.
Each entry is the mean of four experiments.

None of the alcohols used at 50 per cent concentration had any effect on astrovirus or echovirus 11. Table II shows the titres of astrovirus following one minute contact with 70 and 90 per cent concentrations. Propan-1-ol, propan-2-ol and butan-2-ol all failed to reduce the virus titre even when used at 90 per cent concentration. Seventy per cent ethanol produced a 1 log_{10} drop in astrovirus count and a 4 log_{10} drop was obtained with 90 per cent. Methanol was the most effective of all the alcohols tested causing a 3 log_{10} drop in astrovirus titre when used at 70 per cent concentration. Ninety per cent methanol reduced the virus count below the limit of sensitivity of the test (10 infective units/ml reaction mixture). The presence of faecal material adversely affected the action of 90 per cent ethanol but interfered less with methanol.

Echovirus 11 was resistant to the three higher alcohols at the highest concentration tested in the reaction mixture. Seventy-six per cent ethanol caused a 3 log_{10} reduction in virus count and at least a 4 log_{10} drop in titre followed the use of 76 per cent methanol (Table III).

Discussion

Previous studies on the action of disinfectants against the lamb rotavirus (Snodgrass & Herring, 1977) were concerned with those agents suitable for use on inanimate objects. Contaminated fomites may represent one route of cross-infection, but person to person transmission via hands is at least as important Hendley *et al.*, 1973). We have shown that rotaviruses are relatively easily inactivated by the al-

Table III. *Echovirus titre after alcohol treatment (1-min holding time)*

	Alcohol in reaction mixture (per cent) 0 (control)	60	76
Methanol	6·3*	4·3–5·3	1·3–2·3
Ethanol	6·3	6·3	3·3
Propan-1-ol	6·3	6·3	6·3
Propan-2-ol	6·3	6·3	6·3
Butan-2-ol	6·3	6·3	6·3

*Log_{10} infective units/ml reaction mixture.
Each entry is the mean of eight experiments.

cohols tested. Tests performed on this virus, even in the presence of particulate faecal matter showed that a 50 per cent concentration of any of the alcohols except methanol completely removed infective viruses as detected by this system. Methanol was the least active of the alcohols tested but even it caused a 3 $\log_{10}$ reduction in virus titre when used at 50 per cent.

The astrovirus and echovirus 11—a representative of the enterovirus genus—were considerably more resistant to alcoholic inactivation. Curiously, the three higher alcohols—propan-1-ol, propan-2-ol and butan-2-ol—which were the most active against rotavirus, failed to reduce the infectivity of astrovirus or echovirus 11, even at 90 per cent concentration. A 4 $\log_{10}$ fall in astrovirus titre was obtained with 90 per cent ethanol and methanol.

The effects of the alcohols on the enterovirus were similar to their effects on astrovirus. Against both small round viruses, methanol was the most active, although it had performed least well against rotaviruses.

A consideration of these results suggests that in situations where virus infections may be disseminated via the hands, the use of an alcoholic disinfectant in addition to, or as a partial substitute for handwashing, should help limit their spread. For this purpose the broadest spectrum of virucidal activity is desirable, and this, we have shown, is achieved with high concentrations (90 per cent) of either ethanol or methanol.

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